

Rec'd PCT/PTO

03 MAR 2005

REC'D 20 OCT 2003

WIPB PAT

P1 1078591

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

October 15, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE.

APPLICATION NUMBER: 60/408,681

FILING DATE: September 06, 2002

RELATED PCT APPLICATION NUMBER: PCT/US03/27873



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

W. Montgomery
W. MONTGOMERY
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

BEST AVAILABLE COPY


09-09-02 60408681 . 07/14/02

Please type a plus sign (+) inside this box → ☐

PTO/SB/18 (2-01)
Approved for use through 10/31/2002 OMB 0851-0032
Patent and Trademark Office U.S. DEPARTMENT OF COMMERCE

Docket Number: 218278

PROVISIONAL APPLICATION FOR PATENT COVER SHEET
This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

INVENTOR(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
Mark E. Steven A. John R.	Dudley Rosenberg Wunderlich	Silver Spring, Maryland Potomac, Maryland Bethesda, Maryland
<input type="checkbox"/> Additional inventors are being named on the separately numbered sheets attached hereto.		
TITLE OF THE INVENTION (280 characters max)		
IMMUNOTHERAPY WITH <i>IN VITRO</i> -SELECTED ANTIGEN-SPECIFIC LYMPHOCYTES AFTER NONMYELOABLATIVE LYMPHODEPLETING CHEMOTHERAPY		
CORRESPONDENCE ADDRESS		
Direct all correspondence to:		
<input checked="" type="checkbox"/> Customer Number 23460  23460 PATENT TRADEMARK OFFICE	<input type="checkbox"/> Carol Larcher Leydig, Voit & Mayer, Ltd. Two Prudential Plaza, Suite 4900 180 North Stetson Chicago, Illinois 60601-6780 U.S.A.	
ENCLOSED APPLICATION PARTS (check all that apply)		
<input checked="" type="checkbox"/> Specification (including any claims and abstract) Number of Pages: 15	<input type="checkbox"/> Power of Attorney	
<input type="checkbox"/> Drawings Number of Sheets: 0	<input type="checkbox"/> Assignment	
<input checked="" type="checkbox"/> Application Data Sheet. See 37 CFR 1.76	<input type="checkbox"/> CD(s), Number	
	<input type="checkbox"/> Other (specify)	
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT		
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.		
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fee(s).		
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fee(s) or credit any overpayment to Deposit Account Number 12-1216. A duplicate copy of this communication is enclosed for that purpose.		
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any deficiencies in filing fees to Deposit Account Number 12-1216. A duplicate copy of this communication is enclosed for that purpose.		
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government:		
<input type="checkbox"/> No.		
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: National Institutes of Health		

Respectfully submitted,

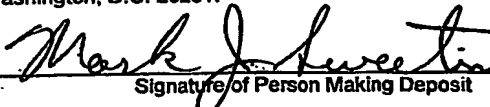

Carol Larcher, Registration No. 35,243
LEYDIG, VOIT & MAYER, LTD.
Two Prudential Plaza, Suite 4900
180 North Stetson
Chicago, Illinois 60601-6780
(312) 616-5600 (telephone)
(312) 616-5700 (facsimile)

Date: September 6, 2002

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this correspondence and the documents referred to as attached or enclosed therein are being deposited with the United States Postal Service on September 6, 2002, in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR 1.10, Mailing Label Number EL 841 133 096 US, addressed to: Commissioner for Patents, Washington, D.C. 20231.


Typed or Printed Name of Person Making Deposit


Signature of Person Making Deposit

Application Data Sheet**APPLICATION INFORMATION**

DHHS No. E-275-02/0

Application Number:: Not Yet Assigned
Filing Date:: September 6, 2002
Application Type:: Provisional
Subject Matter:: Utility
Suggested classification::
Suggested Group Art Unit::
CD-ROM or CD-R?:: None
Number of CD Disks:
Number of Copies of CDs::
Sequence Submission?::
Computer Readable Form (CRF)?:: No
Number of Copies of CRF::

Title:: IMMUNOTHERAPY WITH *IN VITRO*-SELECTED
ANTIGEN-SPECIFIC LYMPHOCYTES AFTER
NONMYELOABLATIVE LYMPHODEPLETING
CHEMOTHERAPY

Attorney Docket Number:: 218278
Request for Early Publication?:: No
Request for Non-Publication?:: No
Suggested Drawing Figure::
Total Drawing Sheets::
Small Entity?:: No
Latin Name::
Variety denomination name::
Petition Included?:: No
Petition Type::
Licensed US Govt. Agency:: National Institutes of Health
Contract or Grant Numbers::
Secrecy Order in Parent Appl.?:: No

APPLICANT INFORMATION

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Mark
Middle Name:: E.
Family Name:: Dudley
Name Suffix::
City of Residence:: Silver Spring
State or Prov. of Residence:: Maryland
Country of Residence:: US
Street of mailing address:: 1605 Cody Drive

City of mailing address:: Silver Spring
State or Province of mailing address:: Maryland
Country of mailing address:: US
Postal or Zip Code of mailing address:: 20902
Inventor Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Steven
Middle Name:: A.
Family Name:: Rosenberg
Name Suffix::
City of Residence:: Potomac
State or Prov. of Residence:: Maryland
Country of Residence:: US
Street of mailing address:: 10104 Iron Gate Road

City of mailing address:: Potomac
State or Province of mailing address:: Maryland
Country of mailing address:: US

Postal or Zip Code of mailing address:: 20854

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: John
Middle Name:: R.
Family Name:: Wonderlich
Name Suffix::
City of Residence:: Bethesda
State or Prov. of Residence:: Maryland
Country of Residence:: US
Street of mailing address:: 10113 Parkwood Terrace

City of mailing address:: Bethesda
State or Province of mailing address:: Maryland
Country of mailing address:: US
Postal or Zip Code of mailing address:: 20814

CORRESPONDENCE INFORMATION

Correspondence Customer Number:: 23460
Phone:: (312) 616-5600
Fax:: (312) 616-5700
E-mail Address:: mail@leydig.com

REPRESENTATIVE INFORMATION

Representative Customer Number:: 23460

Representative Designation:: **Registration Number::** **Representative Name::**

DOMESTIC PRIORITY INFORMATION

Application:: Continuity Type:: Parent Application:: Parent Filing Date::

FOREIGN APPLICATION INFORMATION

Country:: Application Number:: Filing Date:: Priority Claimed

ASSIGNEE INFORMATION

Assignee name:: Government of the United States of America, represented by the
 Secretary, Department of Health and Human Services

Street of mailing address:: 6011 Executive Blvd., Suite 325

City of mailing address:: Rockville

State or Province of
mailing address:: Maryland

Country of mailing
address:: US

Postal or Zip Code of
mailing address:: 20852

IMMUNOTHERAPY WITH *IN VITRO*-SELECTED
ANTIGEN-SPECIFIC LYMPHOCYTES AFTER
NONMYELOABLATIVE LYMPHODEPLETING CHEMOTHERAPY

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the combined use of immunotherapy and chemotherapy to promote the regression of a cancer in a mammal.

BACKGROUND OF THE INVENTION

The immunotherapy of patients with cancer requires the generation *in vivo* of large numbers of highly avid anti-tumor lymphocytes that can overcome normal tolerance and sustain an attack against a solid tumor. Immunization of melanoma patients with cancer antigens can increase the number of circulating CD8+ cytotoxic T-lymphocyte precursor cells (pCTL), but this has not correlated with clinical tumor regression, suggesting a defect in function or activation of the pCTL (Rosenberg et al., Nat. Med 4: 321 (1998)).

Adoptive cell transfer therapy provides the opportunity to overcome tolerogenic mechanisms by enabling the selection and *ex vivo* activation of highly selected T-cell subpopulations and by manipulating the host environment into which the T-cells are introduced. Prior clinical trials, including the transfer of highly active cloned anti-tumor T-cells failed to demonstrate engraftment and persistence of the transferred cells (Rosenberg et al., J. Nat'l. Cancer Inst. 86(15): 1159 (1994); Yee et al., J. Exp. Med. 192: 1637 (2000); Dudley et al., J. Immunother. 24(4): 363 (2001); Dudley et al., J. Immunother. 25(3): 243 (2002)). Lymphodepletion can have a marked effect on the efficacy of T-cell transfer therapy in murine models (Berenson et al., J. Immunol. 115: 234 (1975); Eberlein et al., J. Exp. Med. 156: 385 (1982); North, J. Exp. Med. 155: 1063 (1982); and Rosenberg et al., Science 233: 1318 (1986)) and may depend on the destruction of suppressor cells, disruption of homeostatic T-cell regulation, or abrogation of other normal tolerogenic mechanisms.

The present invention seeks to overcome the differences in the art by providing a combined method of nonmyeloablative lymphodepleting chemotherapy and immunotherapy in which the transferred cells engraft and persist and promote the regression of a cancer. This and other objects and advantages of the present

invention, as well as additional inventive features, will be apparent from the detailed description provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of promoting the regression of a cancer in a mammal. The method comprises (i) administering to the mammal nonmyeloablative lymphodepleting chemotherapy and (ii) (a) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted; and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, by the same route or a different route, a T-cell growth factor that promotes the growth and activation of the autologous T-cells, or (b) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, modified to express a T-cell growth factor that promotes the growth and activation of the autologous T-cells, and rapidly expanded *in vitro* only once, whereupon the regression of the cancer in the mammal is promoted.

Also provided is a method of promoting the regression of metastatic melanoma in a human. The method comprises (i) intravenously administering around 60 mg/kg of cyclophosphamide for two days followed by around 25 mg/m² fludarabine for five days and (ii) subsequently intravenously administering (a) an infusion of around 2.3×10^{10} - 13.7×10^{10} autologous T-cells, which have been previously isolated, selected for highly avid recognition of MART-1, and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, a bolus of about 720,000 IU/kg of IL-2 three times daily until tolerance, or (b) an infusion of around 2.3×10^{10} - 13.7×10^{10} autologous T-cells, which have been previously isolated, selected for highly avid recognition of MART-1, modified to express IL-2, and rapidly expanded *in vitro* only once, whereupon the regression of the metastatic melanoma in the human is promoted.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of promoting the regression of a cancer in a mammal. Desirably, the regression is complete, although one of ordinary skill in the art will appreciate that any degree of regression can be beneficial.

The method can be used to promote the regression of any cancer that expresses an antigen that can be recognized by *in vitro*-selected, autologous T-cells. Examples of such cancers include melanoma, lung carcinoma, breast cancer, colon cancer, prostate cancer, and the like. The method is particularly useful to promote the regression of melanoma, including metastatic melanoma, in a mammal.

The mammal can be any mammal. Preferably, the mammal is a human.

The method comprises (i) administering to the mammal nonmyeloablative lymphodepleting chemotherapy and (ii) (a) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, by the same route or a different route, a T-cell growth factor that promotes the growth and activation of the autologous T-cells, or (b) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, modified to express a T-cell growth factor that promotes the growth and activation of the autologous T-cells, and rapidly expanded *in vitro* only once. The autologous T-cells can be heterogeneous, i.e., phenotypically diverse, e.g., include CD4⁺ T-cells among others, and/or can recognize more than one antigen expressed by the cancer, such as two, three, four, or more antigens. The antigen(s) need not be unique to the cancer.

The nonmyeloablative lymphodepleting chemotherapy can be any suitable such therapy, which can be administered by any suitable route. The nonmyeloablative lymphodepleting chemotherapy can comprise the administration of cyclophosphamide and fludarabine, particularly if the cancer is melanoma, which can be metastatic. A preferred route of administering cyclophosphamide and fludarabine is intravenously. Likewise, any suitable dose of cyclophosphamide and fludarabine can be administered. Preferably, around 60 mg/kg of cyclophosphamide are administered for two days after which around 25 mg/m² fludarabine are administered for five days, particularly if the cancer is melanoma.

The autologous T-cells can be isolated from the mammal by any suitable means as are known in the art and exemplified herein in Example 1. Similarly, selection methods for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, are known in the art and are exemplified herein in Example 1. The autologous T-cells must be rapidly expanded *in vitro* only once, in accordance with methods known in the art and exemplified herein in Example 1. Rapid expansion of T-cell cultures requires non-specific T-cell receptor stimulation in the presence of feeder lymphocytes and IL-2. The non-specific T-cell receptor stimulus can consist of around 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody available from Ortho, Raritan, NJ. If the autologous T-cells are modified to express a T-cell growth factor that promotes the growth and activation of the autologous T-cells, any suitable methods of modification as are known in the art can be used. See, e.g., Sambrook and Russell, *Molecular Cloning*, 3rd ed., SCHL Press (2001). Desirably, modified autologous T-cells express the T-cell growth factor at high levels. T-cell growth factor coding sequences, such as that of IL-2, are readily available in the art, as are promoters, the operable linkage of which to a T-cell growth factor coding sequence promote high level expression.

T-cells can be selected for highly avid recognition of any of the unique antigens produced as a result of the estimated 10,000 genetic mutations encoded by each tumor cell genome. If the cancer is melanoma, such as metastatic melanoma, preferably the T-cells are selected for highly avid recognition of MART-1, gp100, or a "unique" or patient-specific antigen derived from a tumor-encoded mutation. Other suitable melanoma antigens for which highly avid recognition by T-cells can be selected include, but are not limited to, tyrosinase, tyrosinase related protein (TRP)1, TRP2, or MAGE. Antigens, such as NY-ESO-1, telomerase, p53, HER2/neu, carcinoembryonic antigen, or prostate-specific antigen, can be used to select for highly avid recognition by T-cells for treatment of lung carcinoma, breast cancer, colon cancer, prostate cancer, and the like.

The T-cells can be administered by any suitable route as known in the art. Preferably, the T-cells are administered as an intravenous infusion, which preferably lasts approximately 30-60 min. Likewise, any suitable dose of T-cells can be administered. Preferably, from about 2.3×10^{10} T-cells to about 13.7×10^{10} T-cells are administered, with an average of around 7.8×10^{10} T-cells, particularly if the cancer is melanoma.

The T-cell growth factor can be any suitable growth factor that promotes the growth and activation of the autologous T-cells administered. IL-2 is a preferred T-cell growth factor. The T-cell growth factor can be administered by any suitable route. Preferably, the T-cell growth factor, such as IL-2, is administered intravenously as a bolus injection. Desirably, the dosage of the T-cell growth factor, such as IL-2, is what is considered by those of ordinary skill in the art to be high. Preferably, a dose of about 720,000 IU/kg of IL-2 is administered three times daily until tolerance, particularly when the cancer is melanoma. Preferably, about 5 to about 12 doses of IL-2 are administered, with an average of around 9 doses.

In view of the foregoing, the present invention provides a method of promoting the regression of metastatic melanoma in a human. The method comprises (i) intravenously administering around 60 mg/kg of cyclophosphamide for two days followed by around 25 mg/m² fludarabine for five days and (ii) subsequently intravenously administering (a) an infusion of around 2.3×10^{10} - 13.7×10^{10} autologous T-cells, which have been previously isolated, selected for highly avid recognition of MART-1, and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, a bolus of about 720,000 IU/kg of IL-2 three times daily until tolerance, or (b) an infusion of around 2.3×10^{10} - 13.7×10^{10} autologous T-cells, which have been previously isolated, selected for highly avid recognition of MART-1, modified to express IL-2, and rapidly expanded *in vitro* only once, whereupon the regression of the metastatic melanoma in the human is promoted. Preferably, around 7.8×10^{10} T-cells are administered. Preferably, from about 5 to about 12 doses of IL-2 are administered, with an average of around 9 doses. Preferably, the intravenous infusion lasts approximately 30-60 min.

The above method can be adapted to immunodeficiency diseases and autoimmune diseases, such as AIDS, as well as infectious diseases, such as infection with human immunodeficiency virus (HIV).

EXAMPLES

The following examples serve to illustrate the present invention and are not intended to limit its scope in any way.

LVM 218278

6

Example 1

This example describes the effect of prior lymphodepletion on the persistence and function of adoptively transferred cells.

Thirteen HLA-A2 positive patients with metastatic melanoma received immunodepleting chemotherapy with cyclophosphamide (60 mg/kg) for two days followed by fludarabine (25 mg/m²) for five days. On the day following the final dose of fludarabine, when circulating lymphocytes and neutrophils had dropped to less than 20/mm³, rapidly expanded, highly selected, autologous, tumor-reactive (IFN- γ release of greater than 100 pg/ml and at least two times greater than control when stimulated with an HLA-A2-matched melanoma or an autologous melanoma cell line) T-cell cultures (derived from tumor-infiltrating lymphocytes (TIL) obtained by plating 1×10^6 viable cells of a single-cell suspension of enzymatically digested explant of metastatic melanoma into 2 ml of medium containing 6,000 IU/ml of IL-2 (Rosenberg et al. (1994), *supra*; Dudley et al. (2002), *supra*) and maintained at 5×10^5 - 2×10^6 cells/ml until several million T-cells, then screened for tumor cell recognition by cytokine secretion; most active cultures further expanded in IL-2 to a total cell number above 1×10^8 ; followed by one cycle of rapid expansion, using irradiated allogeneic feeder cells, OKT3 antibody and IL-2 (Riddell et al., J. Immunol. Methods 128: 189 (1990)), prior to use) were harvested and pooled for patient intravenous infusion (average of 7.8×10^{10} cells; range of $2.3 - 13.7 \times 10^{10}$ cells) over approximately 30-60 min and high-dose IL-2 therapy (720,000 IU/kg by bolus intravenous infusion every eight hours to tolerance; average of 9 doses; range of 5-12 doses). The patients all had progressive disease refractory to standard therapies, including high-dose IL-2, and eight patients also had progressed through aggressive chemotherapy.

Response was assessed by radiographic measurements and physical examination. A complete response was defined as the complete disappearance of all evaluable disease. A partial response was defined as a decrease equal to or greater than 50% in the sum of the products of perpendicular diameters of all lesions without the growth of any lesion or the appearance of any new lesion. A mixed response was defined as a decrease in the area of some lesions with concurrent growth of other lesions or the appearance of new lesions. Six of the 13 patients had objective clinical responses to treatment and four others demonstrated mixed responses with significant

LVM 218278

7

shrinkage of one or more metastatic deposits. Objective tumor regression was seen in the lung, liver, lymph nodes, and intraperitoneal masses, and at cutaneous and subcutaneous sites. Five patients, all with evidence of concomitant cancer regression, demonstrated signs of autoimmune melanocyte destruction. All patients recovered from treatment with absolute neutrophil counts greater than $500/\text{mm}^3$ by day 11, but slower recovery of CD4 cells as expected following fludarabine therapy (Cheson, J. Clin. Oncol. 13: 2431 (1995)). One patient had a transient respiratory syncytial virus pneumonia during treatment that cleared within one week.

Example 2

This example describes the function and fate of the adoptively transferred T-cells.

T-cell receptor (TCR) expression was examined in the six patients for whom peripheral blood samples were available at one week and approximately one month post-cell transfer, using two-color FACS with an FITC-conjugated CD8-specific antibody and a panel of PE-conjugated β -chain variable region ($V\beta$)-specific antibodies. $V\beta$ expression was highly skewed in five of the six administered TIL, and these same $V\beta$ families were also over-represented in the peripheral blood of the patients at one week after cell transfer. Two patients exhibited prolonged persistence of individual T-cell receptor $V\beta$ families that predominated the T-cell repertoire. Within a few days of cessation of IL-2 therapy following TIL transfer, these two patients exhibited a pronounced lymphocytosis, with one patient having an absolute lymphocytic count (ALC) reaching peak levels in peripheral blood of over 21,000 cells/ mm^3 on day 7 post-cell infusion, and the other patient having an ALC reaching peak levels in peripheral blood of over 16,000 cells/ mm^3 on day 8 post-cell infusion. Only a few $V\beta$ families dominated the T-cell repertoire of the peripheral blood when analyzed with the antibody panel. Peripheral blood lymphocytes (PBLs) from one patient (ALC of 21,000 cells/ mm^3) sampled at the peak of the lymphocytosis were 94% CD8+, of which 63% expressed $V\beta 12$. Even more pronounced skewing of the T-cell repertoire was observed in the peripheral blood of the other patient (ALC of 16,000 cells/ mm^3) sampled at the peak of lymphocytosis, when 96% of the lymphocytes were CD8+, of which 97% expressed $V\beta 7$.

Additional analysis of TCR usage in PBLs was undertaken using RT-PCR with PCR primers that were designed to amplify all V β gene families (McKee et al., J. Immunother. 23: 419 (2000)). Seven days after cell transfer, strong RT-PCR products were seen in PBL from one patient (ALC of 21,000 cells/mm³) for the reactions with V β 12 and V β 14 primers and faint bands from reactions with V β 4, V β 6 and V β 13 primers. PBL from the other patient (ALC of 16,000 cells/mm³) eight days after TIL transfer demonstrated a strong product only in the reaction using the V β 7 primers. Thus, at the RNA and protein levels, individual TCR V β families constituted a majority of the lymphocytes from peripheral blood of both patients one week after TIL transfer.

In order to assess the diversity of the TCR within the over-expressed V β families, the nucleotide sequence of the β -chain V-D-J regions was determined. The V β 12-specific RT-PCR products from one patient (ALC of 21,000 cells/mm³) were cloned, and six clones each from PBL and TIL were found to have identical sequence and to be identical to the V-D-J sequence from MART-1-reactive T-cell clone derived from the TIL. MART-1 is a normal, non-mutated differentiation antigen expressed on melanomas and normal melanocytes (Kawakami et al., PNAS USA 91: 3515 (1994)). The sequence of the V β 7-specific RT-PCR products from the other patient (ALC of 16,000 cells/mm³) also had identical V-D-J sequences, whether derived from PBL, TIL or a MART-1-reactive T-cell clone derived from the TIL. These results demonstrate that clonal, MART-1-reactive, T-cell populations within the TIL infused into these two patients repopulated the immune systems of these patients. Furthermore, these results suggested that the individual clones underwent large numerical expansion *in vivo*. Assuming an average blood volume of 4 liters, one patient (ALC of 21,000 cells/mm³) had more than 5.0×10^{10} circulating V β 12 lymphocytes, while he was infused with only approximately 1.2×10^{10} V β 12 TIL. The other patient (ALC of 16,000 cells/mm³) had at least 5.6×10^{10} circulating V β 7 lymphocytes, while he was infused with 9.5×10^{10} V β 7 TIL. Even without accounting for additional cells within lymphoid tissues or infiltrating into solid tissues, the preponderance of only a single clone in the peripheral blood of these two patients during their lymphocytic episodes was striking.

The MART-1 reactive clones predominated the CD8+ PBL of these two patients for over four months. The lymphocytosis resolved and white blood cell

LVM 218278

9

counts returned to homeostatic levels over the course of several weeks. As measured by V β 12 antibody and by A2/MART-1 tetramer FACS analysis, the MART-1 reactive clone in the patient with an ALC of 21,000 cells/mm³ remained above 60% of the CD8+ lymphocytes for over 123 days. The patient with an ALC of 16,000 cells/mm³ retained the MART-1 reactive V β 7 T-cell at more than 75% of the CD8+ cells for over 159 days from the date of transfer.

The functional status of the MART-1-reactive cells was tested after transfer by comparing the lytic activity of the PBL during the peak of lymphocytosis with PBL prior to infusion and with the infused TIL by cell-mediated lympholysis assay. High levels of specific lysis of MART-1:27-35 peptide pulsed targets and MART-1-expressing HLA-A2+ tumor cell lines were observed in the infused TIL and the post-infusion PBL. Blood smears of PBL from both patients demonstrated that the circulating lymphocytes exhibited an atypical, blastic and highly active morphology, consistent with their direct *ex vivo* lytic function. Additionally, PBL from both patients secreted little or no inflammatory cytokines when stimulated by tumor cell lines; however, overnight activation of post-transfer PBL in IL-2 restored the specific secretion of inflammatory cytokines, including IFN- γ , GM-CSF and TNF- α . These results suggest that the persistent cells may be in an intermediate state of activation, and that appropriate activation signals at the tumor site *in situ* could induce antigen-specific proinflammatory cytokine secretion as well as lytic activity from the persistent T-cell clones.

The ability of transferred cells to traffic to tumor deposits was investigated by analysis of tumor specimens from the two patients obtained by excisional biopsy before treatment and at multiple times after treatment. After treatment, the biopsied specimens contained large areas of necrotic tumor, and areas of dense, diffuse lymphocytic infiltrates. Immunohistochemical staining revealed that the lymphocytic infiltrates consisted predominantly of CD8+ cells. The infiltrating T-cells from the patient with an ALC of 21,000 cells/mm³ were predominantly V β 12, but not V β 7, while T-cells infiltrating tumor tissue from the patient with an ALC of 16,000 cells/mm³ were predominantly V β 7, but not V β 12. RNA from the biopsied specimens of the patient with an ALC of 21,000 cells/mm³ obtained 20 days after cell transfer was analyzed by RT-PCR using the panel of V β -specific primers and V β 12 was a predominant product in two independent tumor specimens (V β 14 was not

LVM 218278

10

evident in either sample). Sequence analysis of the V β 12 V-D-J region from tumor tissue revealed that the β -chain sequence was identical to the V β 12-derived sequence of the TIL, the post-treatment PBL, and the MART-1-specific clone. Both of MHC class I and MHC class II antigens were highly expressed in tumor deposits after therapy, but expressed only at low levels or not at all in tumors prior to TIL treatment. MHC class I and class II antigen expression in tumor cells is indicative of an ongoing inflammatory immune reaction, and IFN- γ is known to induce the expression of these antigens (Boehm et al., Ann. Rev. Immunol. 15: 749 (1997)). Taken together, these results are consistent with trafficking to the tumor of the *in vivo*-expanded V β 12 (patient with ALC of 21,000 cells/mm³) or V β 7 (patient with ALC of 16,000 cells/mm³) TIL, recognition of the MART-1 antigen expressed by the tumor cells, secretion of IFN- γ and other cytokines by the activated lymphocytes, and establishment of an inflammatory anti-tumor immune response within the tumor nodules.

Both patients exhibited significant regression of metastatic melanoma and the onset of anti-melanocyte auto-immunity. One patient (ALC of 21,000 cells/mm³) exhibited regression of more than 95% of his cutaneous and subcutaneous melanoma, and developed vitiligo on his forearms. His metastatic melanoma has shown no sign of recurrence at eight months after treatment. At four months after cell infusion, he developed an EBV-related lympho-proliferative disease (he was EBV sero-negative prior to treatment) that has been reported in EBV sero-negative patients receiving allogeneic transplants (O'Reilly et al., Important Adv. Oncol. 149 (1996)) and is undergoing treatment for this problem. The other patient (ALC of 16,000 cells/mm³) exhibited 99% disappearance of his nodal, cutaneous and subcutaneous melanoma. Fourteen days after cell infusion, during the active regression of melanoma, he developed bilateral acute anterior uveitis characterized by a fibrinous pupillary membrane. This autoimmune manifestation had not been detected in over 600 patients who were treated with high dose IL-2, including many who exhibited objective clinical response to treatment (Rosenberg et al., Ann. Surg. 228: 307 (1998)). He has responded to steroid eye drops to suppress inflammation, and remains healthy with normal vision and without signs of recurrent melanoma over seven months after treatment. Although the absolute lymphocyte counts decayed to

LVM 218278

11

normal levels after 3-4 weeks in both patients, the composition of the resulting lymphocyte pool remained highly skewed.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

LVM 218278

12

WHAT IS CLAIMED IS:

1. A method of promoting the regression of a cancer in a mammal, which method comprises:

(i) administering to the mammal nonmyeloablative lymphodepleting chemotherapy, and

(ii) subsequently administering:

(a) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, by the same route or a different route, a T-cell growth factor that promotes the growth and activation of the autologous T-cells, or

(b) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, modified to express a T-cell growth factor that promotes the growth and activation of the autologous T-cells, and rapidly expanded *in vitro* only once,

whereupon the regression of the cancer in the mammal is promoted.

2. The method of claim 1, wherein the T-cell growth factor is interleukin-2 (IL-2).

3. The method of claim 1 or 2, wherein the nonmyeloablative lymphodepleting chemotherapy comprises the administration of cyclophosphamide and fludarabine.

4. The method of claim 3, wherein around 60 mg/kg of cyclophosphamide are administered for two days after which around 25 mg/m² fludarabine are administered for five days.

5. The method of claim 4, wherein the cyclophosphamide and fludarabine are administered intravenously.

6. The method of any of claims 2-5, wherein a dose of about 720,000 IU/kg of IL-2 is administered three times daily until tolerance.

7. The method of claim 6, wherein from about 5 to about 12 doses of IL-2 are administered.

8. The method of claim 7, wherein around 9 doses of IL-2 are administered.

~~9.~~ 9. The method of claim 6, wherein the dose of IL-2 is administered as a bolus intravenous injection.

10. The method of any of claims 1-19, wherein from about 2.3×10^{10} T-cells to about 13.7×10^{10} T-cells are administered.

11. The method of claim 10, wherein around 7.8×10^{10} T-cells are administered.

12. The method of any of claims 1-11, wherein the T-cells are administered as an intravenous infusion.

13. The method of claim 12, wherein the intravenous infusion lasts approximately 30-60 min.

14. The method of any of claims 1-13, wherein the cancer is melanoma.

15. The method of claim 14, wherein the T-cells bind to melanoma antigen recognized by T-cells-1 (MART-1).

16. The method of any of claims 1-15, wherein the cancer is metastatic.

17. The method of any of claims 1-16, wherein the mammal is a human.

18. A method of promoting the regression of metastatic melanoma in a human, which method comprises:

(i) intravenously administering around 60 mg/kg of cyclophosphamide for two days followed by around 25 mg/m^2 fludarabine for five days, and

LVM 218278

14

(ii) subsequently intravenously administering:

(a) an infusion of around 2.3×10^{10} - 13.7×10^{10} autologous T-cells, which have been previously isolated, selected for highly avid recognition of MART-1, and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, a bolus of about 720,000 IU/kg of IL-2 three times daily until tolerance, or

(b) an infusion of around 2.3×10^{10} - 13.7×10^{10} autologous T-cells, which have been previously isolated, selected for highly avid recognition of MART-1, modified to express IL-2, and rapidly expanded *in vitro* only once, whereupon the regression of the metastatic melanoma in the human is promoted.

19. The method of claim 18, wherein around 7.8×10^{10} T-cells are administered.

20. The method of claim 18 or 19, wherein from about 5 to about 12 doses of IL-2 are administered.

21. The method of claim 20, wherein around 9 doses of IL-2 are administered.

22. The method of any of claims 18-21, wherein the intravenous infusion lasts approximately 30-60 min.

LVM 218278

15

ABSTRACT

A method of promoting the regression of a cancer in a mammal comprising:

(i) administering to the mammal nonmyeloablative lymphodepleting chemotherapy, and

(ii) subsequently administering:

(a) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, and rapidly expanded *in vitro* only once, and, either concomitantly with the autologous T-cells or subsequently to the autologous T-cells, by the same route or a different route, a T-cell growth factor that promotes the growth and activation of the autologous T-cells, or

(b) autologous T-cells, which have been previously isolated, selected for highly avid recognition of an antigen expressed by the cancer, the regression of which is to be promoted, modified to express a T-cell growth factor that promotes the growth and activation of the autologous T-cells, and rapidly expanded *in vitro* only once,

whereupon the regression of the cancer in the mammal is promoted.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.